LOCALIZATION AND PROPERTIES OF PHOSPHOPROTEIN PHOSPHATASE IN THE FROG EGG AND EMBRYO[1]

SYLVAN NASS[2]

Department of Biology, University College of Arts and Science, New York University, New York 53, New York

Phosphoprotein phosphatase (PPPase) has been suggested to be important in the mechanism of yolk utilization in the frog embryo (Harris, 1946; Barth and Barth, 1954; Flickinger, 1956, 1957). Knowledge of the mechanism of regulation of the enzymatic activity during development must follow from a thorough understanding of the properties, location and changes in activity which occur during morphogenesis. In view of the unsettled nature of the literature concerning these areas an investigation has been made into various phases of these problems.

The use of different fractionation and incubation procedures upon ovarian eggs has resulted in PPPase activity being found in the yolk (Harris, 1946; Nass, 1956), the small yolk (Panijel, 1950), the pigment-containing fraction (Barth and Barth, 1954) and in water-soluble dephosphorylated vitellin (Flickinger, 1956). All of these investigators have reported enzymatic activity in several of these fractions of ovarian homogenates. There have been reports on calcium activation of the enzyme (Flickinger, 1956) and on calcium inhibition (Nass, 1956). The phosphate release from vitellin has also been suggested to be non-enzymatic (Flickinger, 1956).

Mezger-Freed (1953) studied the changes in the endogenous phosphate released from embryo homogenates during development and found peak activities just before and during gastrulation and a steady decline after neurulation. Flickinger (1956) reported a stage-wise increase in PPPase activity from eggs to hatched larvae. Barth and Barth (1951) found a low enzymatic activity of KCl extracts until gastrulation when a slight increase was observed. The activity reached its peak at hatching and declined thereafter.

Yolk has also been suggested to play a primary role in morphogenesis. It has been shown that the yolk is important in determining the polarity of the egg (Ancel and Vintemberger, 1948), and Dalcq and Pasteels (1937) have hypothesized that the product of the interaction of a cortical and vitellin gradient determines the fate of the various regions of the embryo. Most microscopical investigations of yolk utilization indicate that the yolk platelets are solubilized most rapidly at the time of hatching, although evidence for an earlier utilization of yolk has also been reported (Voss, 1934; Bragg, 1939).

[1] This paper is condensed from a dissertation presented to the Faculty of the Graduate School of Arts and Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University. Part of the work was supported by a grant from the National Science Foundation to Prof. Paul R. Gross.

[2] Present address: The Wenner-Grens Institute for Experimental Biology, Stockholm, Sweden.

There is excellent agreement that the yolk is first used in the most actively differentiating regions, especially the chorda-mesoderm (Bragg, 1939; Flickinger, 1949, 1954). Flickinger (1949) has correlated the disappearance of yolk with the initiation of pigmentation of neural crest pro-pigment cells, as well as with increased oxygen consumption that follows yolk utilization. Electron microscopy of amphibian eggs and embryos shows that an intimate relationship exists between yolk and mitochondria, and Lanzavecchia and Le Coultre (1958) have suggested that a direct conversion of yolk to mitochondria takes place. Karasaki (1959) has shown that the mitochondria increase in number and complexity from late gastrulation onward, at the time when yolk utilization and oxygen consumption increase.

The major part of this investigation is concerned with clarifying the problem of the localization of PPPase in the ovarian egg and characterizing its properties after freeing the enzyme from its endogenous substrate. The changes in the enzyme activity during development have been investigated and correlated with the localization studies and with the known morphogenetic movements occurring at various stages of development.

Materials and Methods

A. *Preparation of fractions*

1. *Yolk fractions.* Ovaries of *Rana pipiens* were excised, washed and homogenized in cold 0.05 *M* Tris-maleate buffer, pH 6.8, which in some cases contained 0.5 *M* NaCl. The ovary and oocytes were disrupted in one or two strokes of the pestle. Excessive homogenization results in pigment adsorption upon the yolk platelets, as previously described (Panijel, 1950; Nass, 1956). All procedures prior to incubation were performed at temperatures below 4° C. Yolk platelets were isolated according to the method described by Essner (1954), with minor modifications in the centrifugation schedules (Nass, 1961). All preparations were routinely examined microscopically for uniformity and absence of contaminants.

Small yolk platelets were best prepared by removing the bulk of the large yolk at 120 *g* and then centrifuging the supernatant fraction at 700 *g*. The sediment was re-suspended, centrifuged to remove traces of the larger yolk components, and the small yolk was re-sedimented. This procedure was repeated at least five times. In many preparations this small yolk fraction remained gray from pigment adsorption even after ten washings with buffer. Microscopically, the fraction was found to contain few large yolk platelets.

In some instances a combined pigment-small yolk fraction was prepared by centrifugation at 4000 *g* for 10 minutes after previous removal of the large yolk fraction.

A few preparations of large and small yolk by the method described by Panijel (1950) were attempted. In most instances the sucrose medium caused aggregation of the yolk and was not as satisfactory in the separation of the platelets as the method described above. The procedure of centrifuging all the components down at a higher centrifugal force and then re-suspending them also led to excessive, irreversible pigmentation of the yolk fractions.

2. *YP_A and ghosts.* Clean yolk platelets are almost completely and immediately soluble in solutions of ionic strength greater than 0.4 *M*. The small amount of insoluble material (ghosts) was centrifuged down and the soluble yolk re-precipi-

tated by lowering the salt concentration to 0.10 *M* or less by dilution or dialysis against distilled water or dilute buffer, pH 6.8. This material is referred to hereafter as "YP_A" and is equivalent to the YP described by Gross (1954).

3. *YP_B*. When ovaries were homogenized in buffer containing 0.5 *M* NaCl, the first centrifugation was carried out at 10,000 *g* for 15 minutes in order to remove most of the particulate matter while leaving the soluble yolk proteins in the supernatant fluid. The large quantity of lipid-containing material separating centripetally was skimmed off and discarded; the remainder of the supernatant fluid was dialyzed against distilled water or dilute buffer for 24 hours in order to precipitate the yolk proteins. This material is called "*YP_B*" and is similar to the preparations described by Barth and Barth (1954).

4. *Pigmented fraction and extract.* The gummy, black residue after extracting and centrifuging the ovaries was re-homogenized in buffer containing 0.5 *M* NaCl and centrifuged at 4000 *g* for 10 minutes. The procedure was repeated twice in order to remove most traces of adsorbed yolk proteins. This material was sometimes used as the enzyme source, but part of it could be dissolved by extraction with pH 4.9 acetate buffer as described by Barth and Barth (1954). The residue was extracted three times in small volumes of buffer with centrifugation and suspending of the material between each extraction.

5. *Embryonic material.* Eggs and embryos of the desired stage of development (Shumway, 1940) were washed and homogenized in three volumes of 0.1 *M* Tris-maleate buffer, pH 7.2, containing 0.5 *M* KCl. The homogenate was centrifuged and the fractions were prepared as described above.

B. *Storage of fractions*

Yolk platelet organization and properties are disrupted by freezing or acetone powder preparation, and whole ovaries are no longer extractable after freezing. Yolk held at 4° C. releases some phosphate during storage (Panijel, 1950; Nass, 1956). It was found that whole ovaries, intact platelets, yolk platelets soluble at high ionic strengths, and insoluble residues maintained their physical and enzymatic properties for as long as one year when stored in 50% glycerol at −10° C. Material that was prepared for storage was made up in double concentrations of Tris-maleate buffer, pH 6.8, and/or NaCl and then diluted in half with glycerol. For use, the glycerinated material was either diluted or dialyzed against distilled water, centrifuged, washed and re-diluted with the desired buffer.

C. *Substrates*

Phosvitin (Nutritional Biochemical Co.) was used routinely as the substrate because of its favorable solubility properties over the pH range employed. Yolk platelets or solubilized yolk could be used as a substrate after heating at 65–70° C. for three minutes. Most of the enzymatic activity of the yolk was destroyed by this treatment, while the yolk retained its characteristic solubility properties at weak and strong salt concentrations. Heating at higher temperatures irreversibly denatured the proteins.

D. *Analytical procedures*

All fractions were incubated with and without added substrate in both 0.1 *M* and 0.5 *M* NaCl. Controlled variables included enzyme concentration, substrate concentration, ionic strength, pH, temperature, and time of incubation. Most incubations were performed at pH 4.9 for 15 minutes at 28° C. The reaction was stopped by adding 10% trichloroacetic acid (TCA) to yield a final concentration of 7.5%. The samples were centrifuged and an aliquot removed and analyzed for inorganic phosphate by the Fiske-Subbarow procedure, as modified by Leloir and Cardini (1957). The addition of molybdate to a TCA extract of frog eggs or yolk resulted in a clouding of the originally clear extract, as described by Harris (1946) and Mezger-Freed (1953). This necessitated an additional centrifugation before the reducing solution was added.

The total phosphorus method employed was described by Leloir and Cardini (1957), but superoxal (30% hydrogen peroxide) was substituted for nitric acid.

The fractionation of phosphorus-containing compounds was performed by the Schneider (1945) technique.

Soluble protein was sometimes determined by the biuret procedure described by Gornall *et al.* (1949) after standardization of representative samples by the Kjeldahl procedure. Most nitrogen analyses were performed by the micro-Kjeldahl method.

Experiments and Results

The wide variations in the localization of PPPase were studied by comparing the techniques of other investigators (Barth and Barth, 1954; Flickinger, 1956; Panijel, 1950) with our own (Nass, 1956). The experiments reported here are consistent with the view that endogenous PPPase activity of yolk is due to adsorption of pigmented material and that the stability of solubilized yolk is dependent upon the previous steps in the isolation procedure. The described kinetics, pH optima, temperature characteristics, substrate specificity and inhibition studies on fractions freed of endogeneous activity are positive indicators of a true enzymatic hydrolysis of phosphoprotein phosphate.

A. *Observations on yolk fractions prepared by different methods*

The data summarized in Table I show that salt extraction of whole homogenates resulted in a dephosphorylation of the yolk (YP_B) phosphoprotein. A concomitant alteration in the solubility properties of these preparations was also noted. The loss of phosphate from phosphoprotein renders the protein water-soluble (Flickinger, 1956; Nass, 1956). Solubilization of clean yolk (YP_A) followed by centrifugation and re-precipitation by dilution with water showed little loss of phosphoprotein phosphorus, and complete precipitation of the extracted yolk was obtained. Further solubilization and re-precipitation left trace quantities of high-salt-insoluble material in YP_A but large quantities of this material in YP_B. Other evidence of the instability of YP_B phosphoprotein includes the solubility of this material at both acid and alkaline pH as compared with the acid precipitability of YP_A. Upon incubation at pH 4.9, 28° C., YP_A lost phosphate and became water-soluble.

TABLE I

Phosphorus fractions of yolk preparations

	Total P	Acid-soluble P tot. = P_i + true acid-sol.			Lipid P	RNA P	Phospho-protein P	Phospho-protein P + P_i
mg. P/mg. N								
WY*	0.14	0.021	0.017	0.005	0.039	0.014	0.065	0.082
YP_A	0.14	0.026	0.021	0.005	0.045	0.016	0.062	0.083
YP_B	0.12	0.047	0.042	0.005	0.032	0.022	0.015	0.057
% total P								
WY*	100	15	12	3	28	10	46	58
YP_A	100	19	15	4	32	11	44	59
YP_B	100	38	34	4	26	18	12	46

* Whole yolk platelets.

The instability of YP_B phosphoprotein was observed in well buffered (pH 6.8) media and at temperatures not exceeding 4° C., at which PPPase activity is negligible. Other non-enzymatic dephosphorylations have been noted in stored whole yolk (Nass, 1961; Harris, 1946) and in heated yolk where the enzymatic activity had been destroyed. The supernatant fraction of all homogenates contained large quantities of P_i (20% of the total phosphate of the egg), which perhaps were released during the original homogenization procedure.

YP_B preparations of embryonic yolk proteins were found to be much more stable than ovarian YP_B preparations. The endogenous activity of these fractions was much lower, and they were noticeably freer of pigment contaminants than ovarian YP_B.

B. *Localization studies of PPPase in ovarian fractions*

The following experiments provide evidence that PPPase is localized in the pigment fraction and that the endogenous PPPase activity of yolk results from adsorption of pigmented material upon the yolk platelet surface.

(1) The endogenous enzyme activity of similarly prepared yolk fractions was variable when the protein concentrations were equated (Nass, 1956, 1961).

(2) Figure 1 shows that whole yolk platelets, with a very low endogenous activity at low salt concentrations, acted immediately upon added exogenous substrate. YP_A, with the ghost protein removed, had a slightly greater endogenous activity, but a much lower activity upon phosvitin. The isolated ghost fraction was highly reactive against phosvitin. At high salt concentrations, the solubilized whole yolk had a greatly increased endogenous activity, but YP_A activity increased to a much lesser extent. With repeated precipitations, YP_A lost all of its activity against phosvitin and much of its endogenous activity (Nass, 1961).

(3) Table II presents data on the effect of increasing purification of the ovarian fractions upon their PPPase activity. The pigmented fraction retained a constant activity after several washings, while the small and large yolk fractions lost activity with each washing. The ratios of phosphate hydrolysis at low and high salt con-

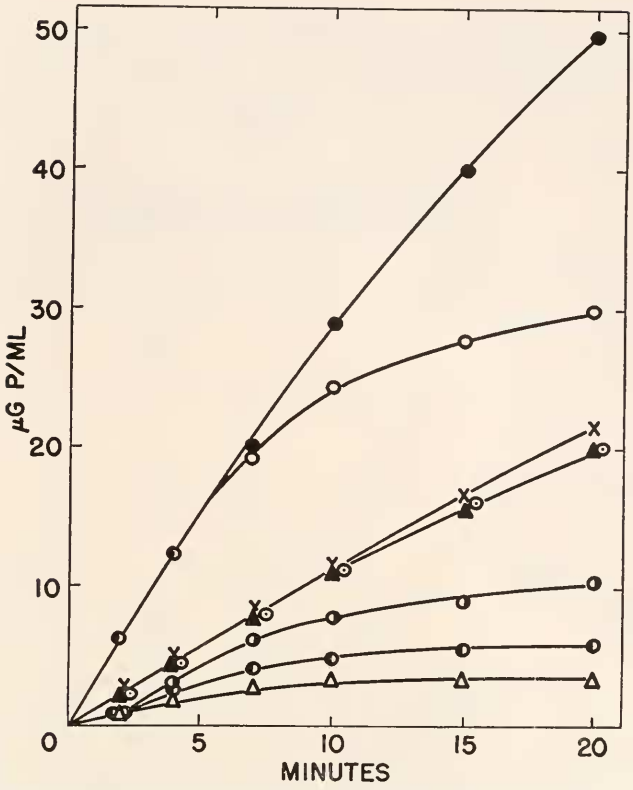

FIGURE 1. Rates of PPPase activity of yolk platelets and YP_A, incubated in 0.1 *M* and 0.4 *M* NaCl, with or without added phosvitin (10 m*M* P). Filled circle: yolk platelets + phosvitin in 0.4 *M* NaCl. Open circle: yolk platelets in 0.4 *M* NaCl. X: YP_A + phosvitin in 0.4 *M* NaCl. Filled triangle: YP_A in 0.4 *M* NaCl. Dotted circle: yolk platelets + phosvitin in 0.1 *M* NaCl. Circle, right half filled: YP_A + phosvitin in 0.1 *M* NaCl. Circle, left half filled: YP_A in 0.1 *M* NaCl. Open triangle: yolk platelets in 0.1 *M* NaCl. Incubated at pH 4.9, 28° C.

centrations show that most of the activity of the large yolk was due to its action on phosvitin in low salt but to its endogenous activity when the yolk was solubilized (see also Figure 1).

Fractionation of ovarian homogenates indicated that only the large and small yolk granules had any endogenous P_i released. Ghosts from the yolk platelets, the pigmented fractions and the supernatant proteins had no endogenous activity (Nass, 1961).

(4) The phosphorus/nitrogen ratio of yolk was 0.14, while the ratio of small yolk was 0.17, ghosts 0.21, and the pigmented fraction 0.28. YP_B fractions had somewhat lower ratios (0.10–0.12) from Shumway (1940) stage 1 through stage 20. Stage 25 YP_B had a distinctly lower ratio (0.08), probably related to new proteins being formed and precipitating with the euglobulin fraction (*e.g.* actomyosin). The increasing PPPase activities of the ovarian fractions against phos-

TABLE II

Comparison of total activities of ovarian fractions

Exp. No.	Fraction	Incubated in 0.1 *M* NaCl		Incubated in 0.4 *M* NaCl	
		(a) Ratio of total activity of fractions against phosvitin minus endogenous activity*	(b) Ratio of the sum of endogenous and phosvitin activities	(a)	(b)
1	Unwashed fractions				
	Pigment-supernatant	1.0	1.0		
	Pigmented small yolk	1.7	1.8		
	Large yolk	3.9	4.7		
2	"Cleaner" fractions				
	Pigment-supernatant	1.0	1.0		
	Pigmented small yolk	1.0	1.0		
	Large yolk	1.9	2.1		
3	"Cleanest" fractions				
	Pigment-supernatant	1.0	1.0	1.0	1.0
	Small yolk	0.7	0.7	0.5	0.7
	Large yolk	1.3	1.4	0.7	1.5

* The total activity of the pigment-supernatant is arbitrarily set at 1.0.

vitin (Figures 2 and 3) were found to parallel their P/N ratios. The exceptional behavior of YP_B having a high PPPase activity against phosvitin (but a lower P/N ratio than yolk) relates to the loss of P_i from YP_B during its preparation. The activity of this fraction may be due to its greater pigment contamination which is evident by the grayness of the preparation, although the P/N ratios would not be markedly altered because of the predominance of the yolk protein.

C. *Properties of PPPase*

1. *Rate of phosphoprotein hydrolysis.* Figure 4 represents the rates of phosvitin hydrolysis by pigment extract. The reaction velocity was linear for 15 minutes, comparable to the rates found for the other fractions discussed previously. (Figs. 2 and 3).

The velocities of P_i release and of protein solubilization from heated yolk by the action of the enzyme are shown in Figure 5. The experimental procedure involved incubation of heated yolk in concentrated salt solutions buffered to pH 4.9. An aliquot was removed for P_i analysis and another aliquot added to 9 volumes of ice-cold water. The precipitate that formed was centrifuged and an aliquot of the supernatant analyzed for protein.

The specific activity of the extract in the presence of optimal concentrations of heated yolk and phosvitin was not higher than with either substrate alone, thus providing evidence for the action of a single enzyme.

Figure 5 shows that the linear rate of phosphate release is only maintained as long as the rate of protein solubilization is linear. In addition, no activity of the extract against heated whole yolk in 0.1 *M* NaCl was found although ghost-free YP_A was solubilized and dephosphorylated under these conditions. These results suggest that disruption of the yolk platelet organization is essential for the action of the enzyme.

Non-protein nitrogen did not increase during incubations with phosphoprotein, indicating that no proteolysis had taken place.

2. *Enzyme concentration.* The results summarized in Figure 6 show that a direct relationship exists between protein concentration and velocity with both phosphoprotein substrates tested. Variations in the salt concentration had little

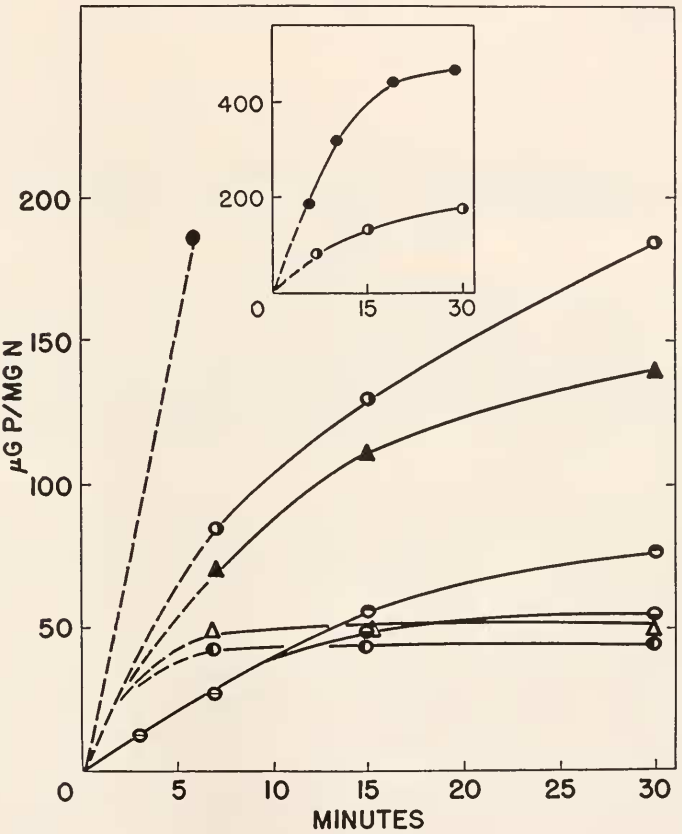

FIGURE 2. Rates of PPPase activity of centrifugal fractions of ovarian homogenates with or without added phosvitin. All incubations in pH 4.9 buffer, 0.4 *M* NaCl, 28° C. Filled circle: pigment + phosvitin. Circle, right half filled: small yolk + phosvitin. Filled triangle: whole homogenate + phosvitin. Circle, upper half filled: large yolk + phosvitin. Circle, lower half filled: large yolk endogenous activity. Open triangle: whole homogenate endogenous activity. Circle, left half filled: small yolk endogenous activity. Inset compares pigment and small yolk activities against phosvitin throughout the 30-minute incubation.

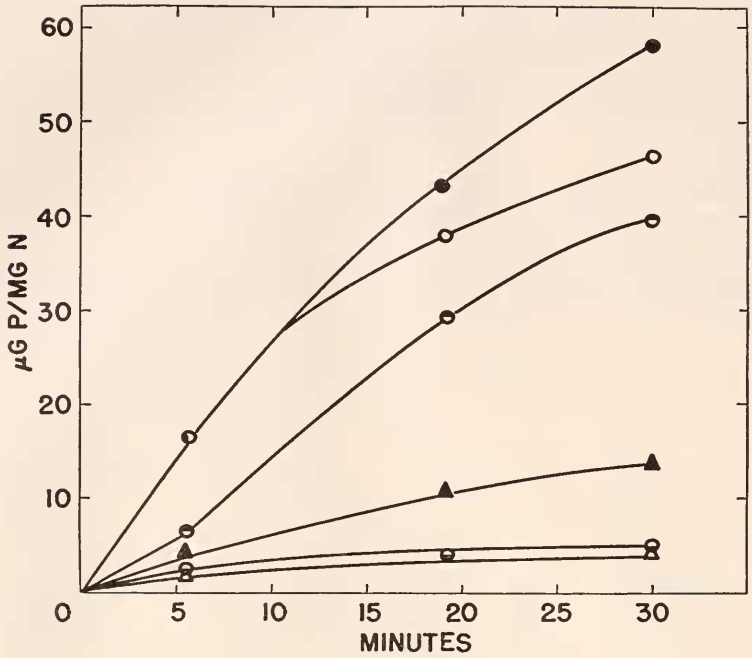

FIGURE 3. Comparison of enzymatic activity of yolk platelets and YP_B in 0.1 *M* and 0.4 *M* NaCl, with or without the addition of phosvitin. YP_B was mostly dephosphorylated prior to incubation, as described in text. Filled circle: yolk platelets + phosvitin in 0.4 *M* NaCl. Open circle: yolk platelet endogenous activity in 0.4 *M* NaCl. Circle, upper half filled: YP_B + phosvitin in 0.4 *M* NaCl. Filled triangle: yolk platelets + phosvitin in 0.1 *M* NaCl. Circle, lower half filled: YP_B endogenous activity in 0.4 *M* NaCl. Open triangle: yolk platelets, endogenous activity in 0.1 *M* NaCl.

effect when the enzyme was incubated with water-soluble phosvitin, whereas heated yolk platelets were an effective substrate only at high salt concentrations. When heated YP_A was used as a substrate, a high hydrolytic rate was found in both weak and strong salt solutions.

3. *Substrate concentration.* Phosvitin, a yolk protein with 9.7% of its weight as phosphorus and a P/N ratio of 0.8 (Mecham and Olcott, 1949), was dissolved in buffer, and the molarity of phosphorus was used as the basis for the substrate concentration curves. Yolk with a P/N ratio of 0.14 required over five times as much protein to reach the same phosphorus concentration. It is apparent from Figure 7 that the phosvitin concentration is nearly optimal at 7 m*M* phosphate, while heated yolk does not approach an optimum until 20 m*M* phosphate. A Lineweaver-Burk analysis (Lineweaver and Burk, 1934) resulted in a Michaelis constant of 3.6 m*M* of phosvitin phosphorus.

Pigment extract had no activity after 15 minutes of incubation against 10 m*M* P concentrations of β-glycerophosphate, disodium phenyl phosphate, and ATP.

4. *Effects of pH.* The results of variations of the pH upon the PPPase activity of pigment extracts with phosvitin as substrate are shown for two temperatures in

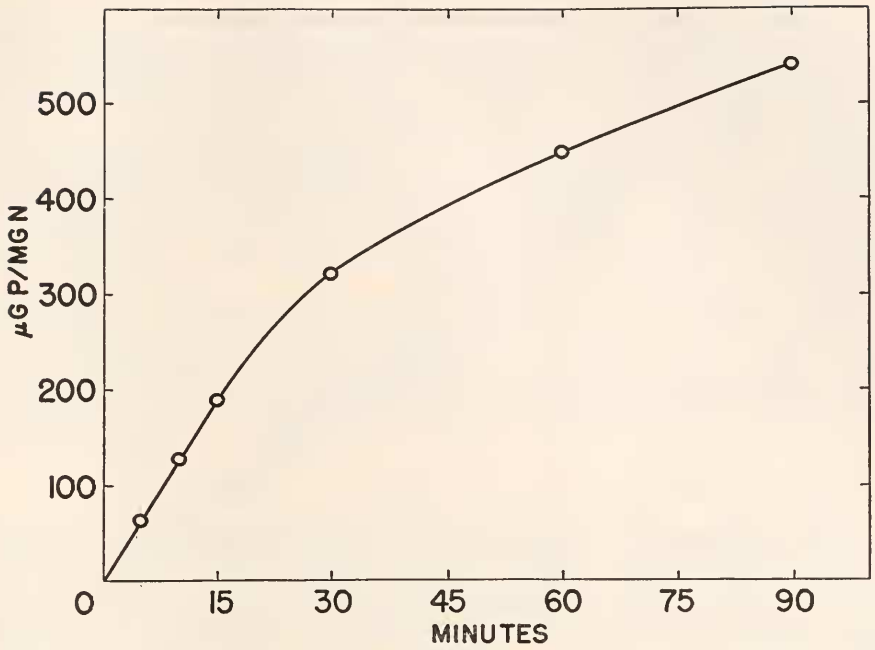

FIGURE 4. Rate of phosphate hydrolysis from phosvitin (10 m*M* P) by pigment extract. Incubated at pH 4.9, 28° C.

Figure 8. The pH curves are very similar to those reported for the endogenous activity of yolk platelets (Nass, 1956) except for a shift of the pH optimum from pH 5.0 in the latter to pH 5.4 with phosvitin.

5. *Effects of temperature.* The activity of the extract against heated yolk in 0.4 *M* NaCl was tested at various temperatures. The temperature optimum for a 10-minute incubation period was found to be at about 50° C., which is similar to that found previously for endogenous yolk activity (Nass, 1956). A sharp decline in the activity occurred from approximately 55° to 65° C. Arrhenius plots of these data and those of a similar experiment with phosvitin as a substrate are shown in Figure 9. Both substrates gave very similar plots with discontinuities at 26° C.

6. *Ionic effects.* Many references have been made throughout this section about the effects of strong salt solutions (0.4 *M* NaCl) and of weak salt solutions (0.1 *M* NaCl). In summary, the activity of the extract upon yolk platelets is minimal in 0.1 *M* NaCl but much greater in 0.4 *M* NaCl. The effects of the ionic strength of the medium on the action of the extract upon phosvitin are very small between 0.4 and 0.1 μ, but a 20% decrease of activity was measured when the ionic strength was lowered to 0.02 μ.

Ca^{++} and Mg^{++} completely inhibited the P_i release at 10^{-1} *M* concentrations but were without effect at lower concentrations. Cu^{++}, Hg^{++}, Mn^{++}, Fe^{+++}, and Al^{+++} completely inhibited the P_i release at 10^{-1} *M* concentrations, and exhibited inhibitions of 75% and 15% at 10^{-2} *M* and 10^{-3} *M*, respectively. Most of the effects were a resultant of precipitation of the reactants at high ionic concentrations.

7. Inhibitors. Both the endogenous and exogenous activities of yolk fractions were markedly inhibited by ammonium molybdate, suggesting that both reactions were enzymatically similar. The Lineweaver-Burk plot of molybdenum inhibition of PPPase extracted from the pigment fraction showed this polyanion to be a non-competitive inhibitor of the enzyme. The ammonium molybdate was observed to precipitate a very small quantity of non-TCA-precipitable material when added to an incubation mixture. The clouding that was usually found when molybdate was added during the Fiske-Subbarow procedure was not observed when this reagent was added to molybdate-inhibited incubation mixtures after TCA precipitation. Inhibition was 50% at 10^{-4} *M* molybdenum.

Other reagents that were reported to be activators and inhibitors of mammalian PPPase (Feinstein and Volk, 1949; Norberg, 1950; Sundararajan and Sarma, 1954; Paigen, 1958), including thioglycollic acid, desoxycholate, ascorbic acid, hydrogen peroxide, and phenanthroline, did not affect frog PPPase at concentrations of 10^{-2} *M*. Oxalate, iodoacetate, cyanide, and fluoride were equally ineffective.

D. *Changes in PPPase activity during development of frog embryos*

Figure 10 shows that the amount of YP_B protein per embryo decreased sharply after stage 20 (hatching). Pigment residues, obtained from ovaries and embryos at different stages of development, were incubated individually with YP_B of stage 2, stage 4, stage 10, stage 20, and with phosvitin. The activities of each residue

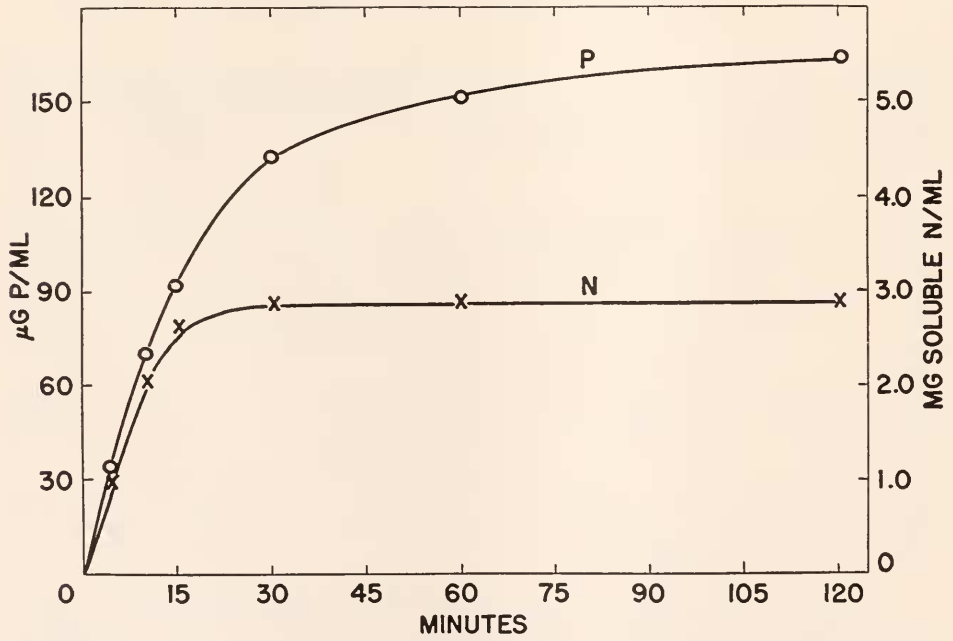

FIGURE 5. Rate of phosphate hydrolysis and protein change from a water-precipitable to a water-soluble state by the action of pigment extract upon heated yolk platelets in 0.4 *M* NaCl, pH 4.9, 28° C.

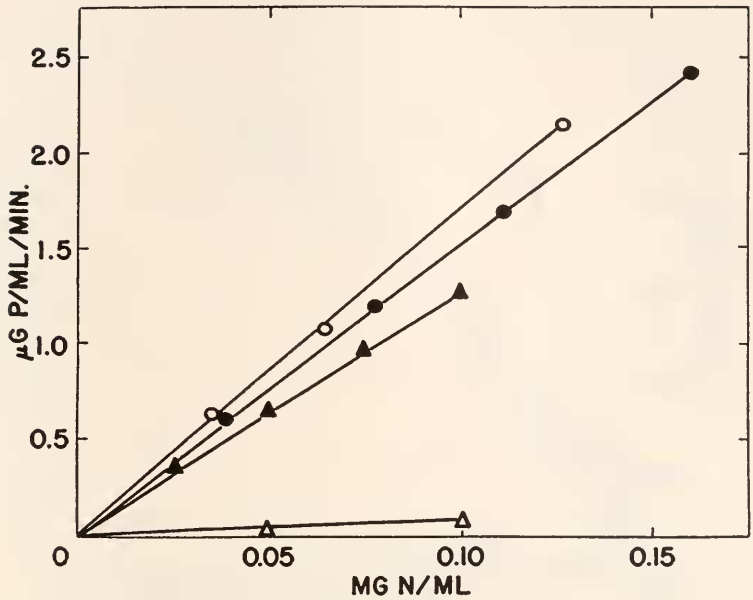

FIGURE 6. The proportionality of the reaction rate to enzyme concentration. Substrates: Open circle: heated yolk platelets (20 m*M* P) in 0.4 *M* NaCl. Filled circle: phosvitin (10 m*M* P) in 0.4 *M* NaCl. Filled triangle: phosvitin in 0.1 *M* NaCl. Open triangle: heated yolk platelets in 0.1 *M* NaCl. Incubation period 15 minutes, pH 4.9, 28° C.

against all of these substrates were similar, and the average value obtained from the incubation of each residue with the five substrates is reported in the figure. Although ovarian pigment had a specific activity of 21 μg. P/mg. N/min., the residues after fertilization had very low activities until stage 17 (tailbud), when the activity sharply increased to the value found for ovarian pigment. The decline of the activities of the residues after hatching paralleled the decline of YP_B extractable from embryos. It should be re-emphasized that the activities of the residues declined after stage 20 when YP_B of stage 2 through stage 20 as well as phosvitin was used as substrate, and therefore the decline was not a result of the smaller quantities of substrate present in embryos after stage 20.

DISCUSSION

The contradictory reports on the localization of the enzyme phosphoprotein phosphatase in the frog egg appear to be a result of the different preparatory techniques, which lead to variable amounts of adsorption artifacts or to non-enzymatic dephosphorylation of the yolk proteins during preparation. Yolk proteins prepared by homogenization and extraction of ovaries at high ionic strength are largely dephosphorylated during the preparation and therefore lose their characteristic euglobulin properties (Table I). This property is not exhibited by yolk proteins prepared by similar extraction techniques of whole yolk platelets. No water-soluble livetin is found when isolated, clean yolk platelets are solubilized and re-precipitated. This result is in agreement with that reported by Ringle (1960).

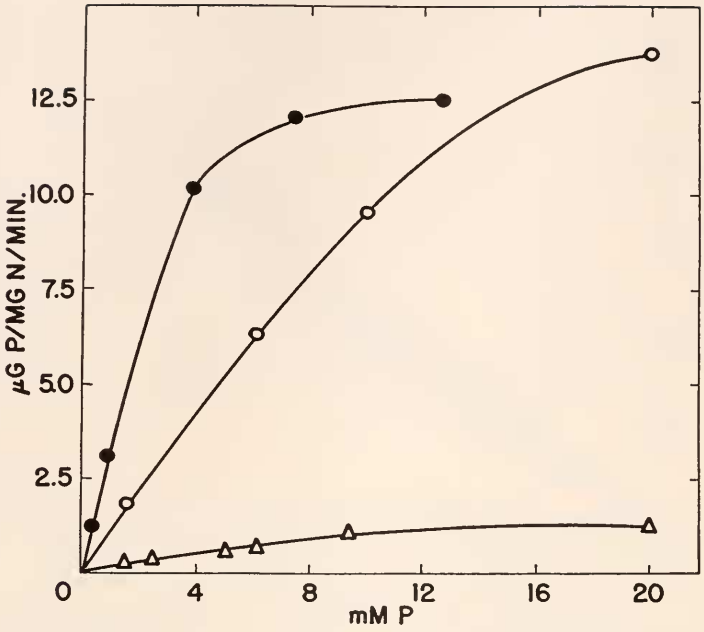

FIGURE 7. Effect of substrate concentration upon enzyme activity. Filled circle: phosvitin in 0.4 *M* NaCl. Open circle: heated yolk platelets in 0.4 *M* NaCl. Open triangle: heated yolk platelets in 0.1 *M* NaCl. Incubation period 15 minutes, pH 4.9, 28° C.

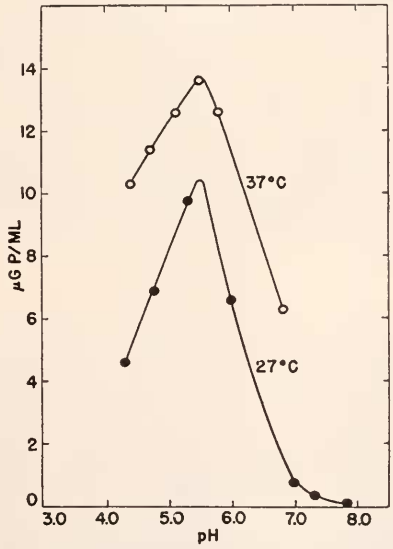

FIGURE 8. The pH dependence of phosphate release. Phosvitin as substrate. Incubation period 10 minutes, 0.05 *M* acetate buffers.

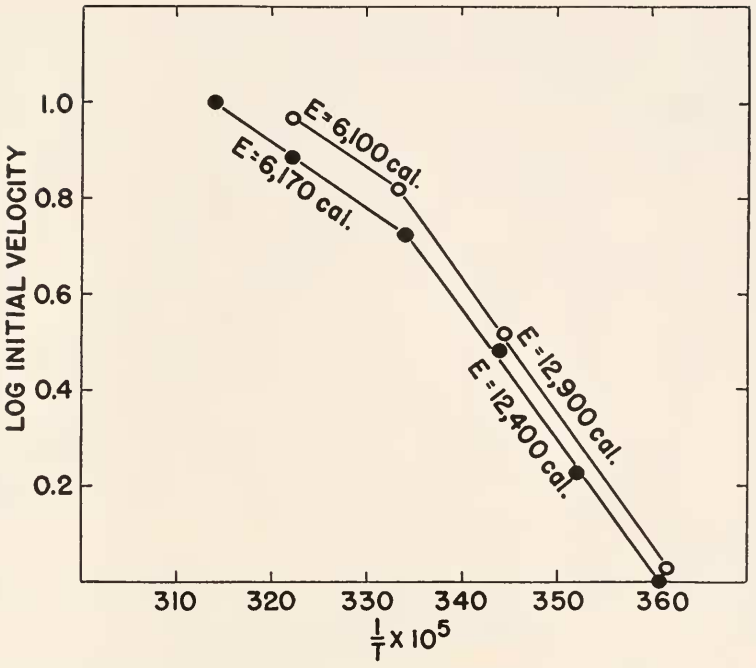

FIGURE 9. Arrhenius plots for PPPase reaction. Upper curve: phosvitin (10 m*M* P) as substrate. Lower curve: heated yolk platelets (20 m*M* P) as substrate. Incubations in 0.4 *M* NaCl, pH 4.9. Incubation period, 10 minutes.

The heavily contaminated YP_B preparations also had a higher RNA phosphate content than clean yolk fractions (Table 1), and it is suggested that the small quantities of RNA measured in all the yolk fractions may be due to adsorption of nucleic acids.

Properties of yolk, such as P/N ratios, the presence or absence of enzymes, the number of electrophoretic, ultracentrifugal and serological components, may need re-examination because of the instability of the material prepared for characterization. The wide variations in P/N ratios reported (0.05 to 0.14) (McClendon, 1910; Fauré-Fremiet and du Streel, 1921; Lawrence *et al.*, 1943; Panijel, 1950; Gross and Gilbert, 1956; Ringle, 1960) may be related to whether the yolk was carefully cleaned before solubilization and re-precipitation or was first extracted and then re-precipitated. The presence of PPPase reported by L. J. Barth (1956) in yolk extracts may be correlated with the adsorption of pigment and with non-enzymatic dephosphorylation. The variable number of electrophoretic, serological and ultracentrifugal components reported for yolk may also be correlated with the dissociation of the yolk proteins following dephosphorylation. Barth and Barth (1954) estimated that the non-phosphoprotein component of yolk made up approximately 90% of the total protein of the yolk. The possibility that part of their yolk preparations was dephosphorylated during preparation is suggested by the results of this study. Thus, the YP_B preparations were found to be unstable and dephos-

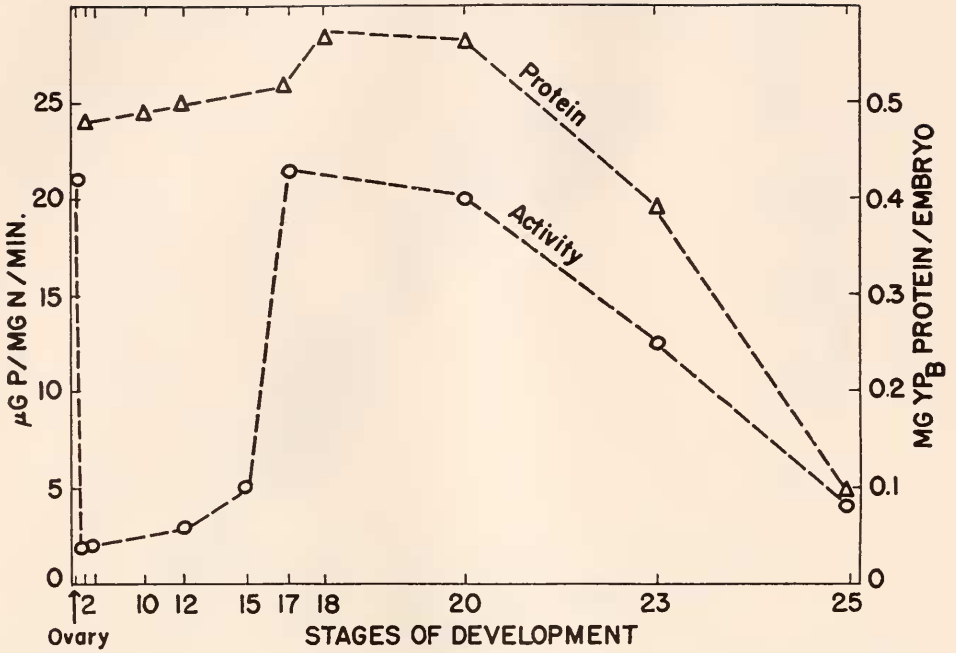

FIGURE 10. Changes in the specific activity of PPPase in pigment residues during embryonic development, relative to the changes in salt-extractable, water-precipitable protein. Abscissa: stages of development (Shumway) superimposed upon an hour of development scale. Activity values are average values, as described in the text. Incubation period, 10 minutes. Buffer, pH 4.9, containing 0.4 *M* NaCl; 28° C.

phorylated during preparation. Further, the YP_B is largely acid-soluble while YP_A is mostly acid-precipitable. Finally, the required pH for solubilization of yolk (pH 11.5) will partly dephosphorylate the yolk non-enzymatically (Flickinger, 1956). The ultracentrifugal and electrophoretic analyses of Schjeide *et al.* (1955) involved a concentrated salt extraction from a pigment-yolk mixture and thus may contain large quantities of water-soluble dephosphorylated vitellin (livetin). Mixing of a preparation of livetin with a preparation of vitellin (YP_B of this report) gave a single electrophoretic peak. The small 6S component (phosphoprotein) and the much larger 11S component (non-phosphoprotein) found by ultracentrifugal analyses (Flickinger and Schjeide, 1957) may also be related to partial dephosphorylation of the preparations prior to analysis. That livetin had other heavier components, 16S and 19S (Schjeide *et al.*, 1955), is suggestive of the report by Gross and Gilbert (1956) that calcium solubilized yolk aggregates with time and thereby changes its sedimentation from 11S to 50–60S. The immunological analyses of Ringle (1960) indicated that the number of apparent protein antigens present in yolk can be changed by altering the salt concentration, the ionic species, or by freezing and thawing. The observations described suggest that the yolk protein is very unstable after it is solubilized, and caution must be used in interpretation of the results of analyses of these preparations.

The conclusions of Ringle (1960) based on micromanipulation, serological and electron microscopic techniques, that the ghost material is not a normal feature of the yolk platelet, are in agreement with the enzymatic studies performed in the present investigation. The ghost protein had a high activity against phosvitin and probably accounts for the activity previously reported to be present in the yolk (Harris, 1946; Panijel, 1950; Nass, 1956). Thus, whole yolk platelets in dilute salt solutions acted immediately on phosvitin, although they had little endogenous activity, while solubilized yolk had no additional activity in the presence of phosvitin (Fig. 1). The P/N ratios of yolk, ghost proteins and pigment further suggest the closer relation of the gray ghost material to the black pigment fraction. The small yolk platelets, having a greater surface/volume ratio, would absorb more of the pigment material, which would thus account for the greater PPPase activity reported (Panijel, 1950) for this fraction as compared with the large yolk granules. Other evidence supporting the view that the ghost surface of the yolk is largely a pigment contaminant and not a regular feature of yolk includes the finding that removal of the ghost material decreased the endogenous activity of the yolk protein and eliminated its action against phosvitin. Further, Table II shows that although the yolk fractions contained the greater total activity, the activity declined markedly with repeated washing of the yolk fractions, but washing did not alter the activity found in the pigment fraction. The reported inability to standardize the enzyme activity of different yolk preparations by measuring the protein content of samples (Nass, 1956) undoubtedly reflects variable quantities of pigment adsorbed to different yolk preparations.

The properties of the enzyme have not previously been studied in preparations free of endogenous activity and in solution. The reported characteristics of the endogenous activity (Nass, 1956) are similar to the properties measured with exogenous substrates and with the enzyme in solution. The equivalent activity of pigment extract on phosvitin at low and high ionic strengths indicates that the salt concentration is a factor of importance only in the solubilization of the yolk proteins and not in activation of the enzyme. The reported activation of the enzyme by Ca^{++} (Flickinger, 1956) may be related to the solubilization of the yolk that is a prerequisite for enzymatic activity against yolk. No activators were found and only molybdate ions out of many agents tested acted as an inhibitor. Except for the mammalian PPPase inhibition by molybdate, the properties of frog PPPase do not resemble any of those reported for the mammalian enzyme (Sundararajan and Sarma, 1954, 1959; Paigen, 1958). The amphibian enzyme is more substrate-specific, no enzymatic action being observed under standard incubation procedures when β-glycerosphosphate, disodium phenyl phosphate or ATP are used as substrates (Nass, 1961).

The experiments reported on the embryonic YP_E preparations show that this material is much more stable than ovarian YP_E. The preparations are noticeably cleaner than ovarian YP_E, and they have negligible endogenous activity. Figure 10 shows that at fertilization the pigment residues lose all their PPPase activity against all the phosphoprotein substrates and that the activity of the residues did not increase again until late gastrulation. The activity reached a maximum at tail-bud stage, where the specific activity was found to be equivalent to that found for the ovarian pigment fraction. The specific activity of the pigment residues

dropped sharply after hatching, paralleling the decline in the quantity of yolk. The water-soluble protein of the frog embryo (which is probably dephosphorylated yolk) increases at neurulation (Gregg and Ballentine, 1946; M. Nass, 1961), which is the approximate time at which PPPase activity increases in the embryo. Fertilization thus apparently inhibits the enzymatic activity found in the pigment granule fraction of the unfertilized egg, and concomitantly the YP_B preparations are cleaner and more stable.

There is clear agreement that yolk utilization is correlated with differentiation and that the most rapidly differentiating areas are the locations for the earliest yolk solubilization (Bragg, 1939; Flickinger, 1949). Flickinger (1956, 1957) and Holtfreter (1947) have maintained that yolk solubilization is of primary importance in inducing neural structures, since ventral explants can be neuralized by treatments which solubilize the yolk proteins. The localization of PPPase in the pigment fraction (probably composed mostly of cortical material) adds support to these views and to the cortical-vitellin gradient hypothesis of Dalcq and Pasteels (1937). Pasteels (1951) suggested that at fertilization the precursor of the active principle of the organization center can be displaced, but after the gray crescent is formed the precursor is "fixed" in the cortical areas and cannot be displaced by centrifugation. This may be compared with the high activity found for PPPase in the homogenized unfertilized egg and with the loss of activity of the pigmented fraction shortly after fertilization. The "fixation" of the cortex would then correspond to the loss of activity of the cortical pigment fraction.

The first area of contact of the cortex with the yolk occurs at the dorsal lip of the blastopore. Although Figure 10 does not indicate more than a very sight rise in the early activity of PPPase, it may become more apparent if experiments were performed upon dissected fragments of this cortical area. The "organizer" action of the dorsal lip could then be interpreted as freeing the yolk-hydrolyzing mechanism, which would provide the necessary materials for neuralization. Experimental neuralization would then involve supplying hydrolyzed yolk proteins to cells which have the capacity but lack the material for neuralization.

Curtis (1960) has shown that the cortical material of cleaving *Xenopus* eggs possesses morphogenetic properties which may be transferred with it; however, the cortical material of the unfertilized egg inhibits development when grafted to a fertile egg. This is also suggestive of a change in the cortex that occurs upon fertilization. Mezger-Freed (1953) studied PPPase activity in the frog and reported that dorsal portions of gastrulae had a greater activity per unit of nitrogen but a lower total activity than ventral fragments. Table II also shows the pigmented fraction to have the greatest activity per unit of nitrogen, but the yolk fraction (mostly vegetal material) had the greatest total activity in unfertilized eggs. These results suggest that the enzyme is located in the dorsal part of the egg cortex, but homogenization disrupts the spatial orientation and thus allows for greater enzyme-substrate interaction.

The localization of the enzyme in the pigment fraction also suggests that the controlling mechanism of yolk utilization is the morphogenetic movement which brings the cortex in contact with the yolk. The leading edge of the invaginating cortex is the area of most active differentiation, and it is of interest that this area is continually in contact with the yolk.

I am grateful to Professor Paul R. Gross for his sponsorship and encouragement throughout this investigation. It is a pleasure to acknowledge the hospitality and the facilities provided by the laboratories at University Heights, New York University, and the Marine Biological Laboratory at Woods Hole, Mass. I am also grateful to Dr. Margit Nass for donating the embryo material, for many suggestions and for her criticism of the manuscript.

Summary

1. The phosphoprotein phosphatase activity of various centrifugal fractions and extracts of homogenates of frog ovaries and embryos was studied in the presence and absence of added phosphoprotein substrates and as a function of a number of environmental variables, including incubation time, pH, temperature and ionic concentrations.

2. Yolk prepared in low ionic strength media was far more stable than yolk extracted from whole homogenates with concentrated salt solutions followed by precipitation by dilution or dialysis. The latter fraction lost its characteristic euglobulin properties during preparation, and this was correlated with the loss of phosphoprotein phosphorus from this material.

3. The rate of endogenous P_i release from yolk platelets was found to be a function of the rate of solubilization of the platelets, and the rate of P_i release was linear only as long as the rate of increase of water-soluble protein was linear.

4. The pigment-containing centrifugal fraction was found to have the highest PPPase activity when ovarian fractions were incubated in the presence of added phosphoproteins. Evidence is presented that the activity found in other centrifugal fractions and extracts is a result of varying degrees of pigment contamination.

5. It was found possible to study the enzyme characteristics of PPPase with the enzyme in solution and in the absence of endogenous substrate.

6. Yolk extracts from embryos were far more stable after re-precipitation by water than similarly prepared ovarian extracts. The enzyme activity of the pigment-containing fraction was found to drop precipitously after fertilization, slowly increase from gastrulation to neurulation and rise to a maximum at tailbud. The activity declined after hatching.

7. The possible sources of the discrepancies in the literature concerning the site of PPPase and the composition of yolk proteins are discussed. The significance of the localization studies and the variations in enzyme activity found during embryogenesis is discussed in relation to the morphological changes occurring during that period.

LITERATURE CITED

Ancel, P., and P. Vintemberger, 1948. Recherches sur le determinisme de la symétrie bilaterale dans l'oeuf des amphibiens. *Bull. Biol. France et Belgique,* **31**: 1–182.

Barth, L. G., and L. J. Barth, 1951. The relation of adenosine triphosphate to yolk utilization in the frog's egg. *J. Exp. Zool.,* **116**: 99–122.

Barth, L. G., and L. J. Barth, 1954. The Energetics of Development. Columbia Univ. Press, New York.

Barth, L. J., 1956. Selective inhibition of cleavage in different regions of the frog egg by sulfhydryl inhibitors. *J. Embryol. Exp. Morph.,* **4**: 73–92.

Bragg, A. H., 1939. Observations upon amphibian deutoplasm and its relation to embryonic and early larval development. *Biol. Bull.*, **77**: 268–284.

Curtis, A. S. G., 1960. Cortical grafts in *Xenopus laevis*. *J. Embryol. Exp. Morph.*, **8**: 163–173.

Dalcq, A., and J. Pasteels, 1937. Une conception nouvelle des bases physiologiques de la morphogenèse. *Arch. Biol.*, **48**: 669–710.

Essner, E. S., 1954. The breakdown of isolated yolk granules by cations. *Protoplasma*, **43**: 79–89.

Fauré-Fremiet, E., and V. du Streel, 1921. Les constituants chimiques de l'oeuf et leur rôle dans le developpement embryonnaire chez la grenouille rousse (*Rana temporaria*). *Bull. Soc. Chim. Biol.*, **3**: 476–482.

Feinstein, R. N., and M. E. Volk, 1949. Phosphoprotein phosphatase in mammalian tissues. *J. Biol. Chem.*, **177**: 339–346.

Flickinger, R. A., 1949. A study of the metabolism of amphibian neural crest cells during their migration and pigmentation *in vitro*. *J. Exp. Zool.*, **112**: 465–484.

Flickinger, R. A., 1954. Utilization of $C^{14}O_2$ by developing amphibian embryos, with special reference to regional incorporation into individual embryos. *Exp. Cell Res.*, **6**: 172–180.

Flickinger, R. A., 1956. The relation of phosphoprotein phosphatase activity to yolk platelet utilization in the amphibian embryo. *J. Exp. Zool.*, **131**: 307–322.

Flickinger, R. A., 1957. The relation between yolk utilization and differentiation in the frog embryo. *Amer. Nat.*, **91**: 373–380.

Flickinger, R. A., and O. A. Schjeide, 1957. The localization of phosphorus and the site of calcium binding in the yolk protein of the frog's egg. *Exp. Cell Res.*, **13**: 312–316.

Gornall, A. G., C. J. Bardawill and M. M. David, 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.*, **177**: 751–766.

Gregg, J. R., and R. Ballentine, 1946. Nitrogen metabolism of *Rana pipiens* during embryonic development. *J. Exp. Zool.*, **103**: 143–168.

Gross, P. R., 1954. On the mechanism of the yolk-lysis reaction. *Protoplasma*, **43**: 416–428.

Gross, P. R., and L. I. Gilbert, 1956. Chemistry and ultrastructure of amphibian yolk platelets. *Trans. N. Y. Acad. Sci., Ser. II*, **19**: 108–117.

Harris, D. L., 1946. Phosphoprotein phosphatase, a new enzyme from the frog egg. *J. Biol. Chem.*, **165**: 541–550.

Holtfreter, J., 1947. Neural induction in explants which have passed through sublethal cytolysis. *J. Exp. Zool.*, **106**: 197–222.

Karasaki, A., 1959. Electron microscopic studies on cytoplasmic structures of ectoderm cells of the *Triturus* embryo during the early phase of differentiation. *Embryologia*, **4**: 247–272.

Lanzavecchia, C., and A. le Coultre, 1958. Origine dei mitocondri durante lo suiluppo embrionale di *Rana esculenta*. Studio al microscopio elettronico. *Arch. Ital. Anat. Embryol.*, **63**: 445–458.

Lawrence, A. S. C., M. Miall, J. Needham and S. Shen, 1943. Studies on the anomalous viscosity and flow birefringence of protein solutions. II. On dilute solutions of proteins from embryonic and other tissues. *J. Gen. Physiol.*, **27**: 233–271.

Leloir, L. F., and C. E. Cardini, 1957. Characterization of phosphorus compounds by acid lability. *In:* Methods in Enzymology. Vol. III. S. P. Colowick and W. O. Kaplan, (eds.), Academic Press, Inc., New York, pp. 843–844.

Lineweaver, H., and D. Burk, 1934. The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.*, **56**: 658–666.

McClendon, J. F., 1910. On the nucleo-albumin in the yolk platelet of the frog's egg, with a note on the black pigment. *Amer. J. Physiol.*, **25**: 195–198.

Mecham, D. K., and H. S. Olcott, 1949. Phosvitin, the principal phosphoprotein of the egg yolk. *J. Amer. Chem. Soc.*, **71**: 3670–3679.

Mezger-Freed, L., 1953. Phosphoprotein phosphatase activity in normal, haploid and hybrid amphibian development. *J. Cell. Comp. Physiol.*, **41**: 493–517.

Nass, M. K., 1961. A comparative study of embryonic and adult frog actomyosin. Thesis, Columbia University, New York.

Nass, S., 1956. Phosphoprotein phosphatase in amphibian yolk. *Trans. N. Y. Acad. Sci., Ser. II*, **19**: 118–129.

NASS, S., 1961. Localization and properties of phosphoprotein phosphatase in the frog egg. Thesis, New York University, New York.

NORBERG, B., 1950. Phosphoprotein phosphatase in the rat. *Acta Chem. Scand.*, **4**: 1206–1215.

PAIGEN, K., 1958. The properties of particulate phosphoprotein phosphatase. *J. Biol. Chem.*, **233**: 388–394.

PANIJEL, J., 1950. L'organisation du vitellus dans les oeufs d'amphibiens. *Biochem. Biophys. Acta*, **5**: 343–357.

PASTEELS, J., 1951. Centre organisateur et potentiel morphogenetique chez les batraciens. *Bull. Soc. Zool. de France*, **76**: 231–271.

RINGLE, D. A., 1960. Organization and composition of amphibian yolk platelets. Thesis, New York University, New York.

SCHJEIDE, O. A., E. LEVI AND R. A. FLICKINGER, 1955. A study of the yolk proteins of frog eggs by physical and chemical means. *Growth*, **19**: 297–306.

SCHNEIDER, W. C., 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.*, **161**: 293–303.

SHUMWAY, W., 1940. Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.*, **78**: 139–147.

SUNDARARAJAN, T. A., AND P. S. SARMA, 1954. Purification and properties of phosphoprotein phosphatase from ox spleen. *Biochem. J.*, **56**: 125–230.

SUNDARARAJAN, T. A., AND P. S. SARMA, 1959. Substrate specificity of phosphoprotein phosphatase from spleen. *Biochem. J.*, **71**: 537–544.

VOSS, N., 1934. Der histochemische Nachweis einer Nukleinsäuresynthese in der Frühentwicklung der Amphibien nebst Bemerkungen über Bedeutung des Dotters als Quelle induzierender Substanzen. *Zeitschr. Mikr. Anat. Forsch.*, **34**: 283–313.